Inhibition of Neutrophil Elastase in CF Sputum by L-658,758

DIANNE D. REES, JOSEPH D. BRAIN, MARY ELLEN WOHL, JOHN L. HUMES and RICHARD A. MUMFORD

ABSTRACT

Elastases in cystic fibrosis (CF) pulmonary fluids damage lung tissue and perpetuate cycles of infection, inflammation and injury. Elastases from three different sources may be present in CF airways: neutrophils, macrophages and Pseudomonas. We measured how well the cephalosporin-based antielastase L-658,758 blocks the activity of human neutrophil elastase (NE), human proteinase-3, human macrophage metalloelastase, mouse macrophage metalloelastase and Pseudomonas aeruginosa elastase. We also examined the ability of L-658,758 to block elastases in CF sputum in vitro. Sputum samples from adult CF patients were fractionated to obtain the aqueous sol phase. These were then studied individually or pooled. Elastolytic activity, which ranged from 3.2 μg elastin degraded/ml sol/min to 26.3 μg elastin degraded/ml sol/min, was measurable in every individual sol sample and in the pooled sol. L-658,758 effectively inhibited elastinolysis by NE, proteinase-3 and the pooled sol but did not inhibit the activity of the metalloelastases, human and mouse macrophage metalloelastase and Pseudomonas elastase. Secretory leukoprotease inhibitor, which inhibited NE but did not inhibit proteinase-3, blocked 90% of sol elastinolytic activity; this suggests that the majority of this activity in the pooled sol derived from NE. L-658,758 was an effective inhibitor of sol elastase, blocking more than 97% of elastinolytic activity in the individual sol samples. We conclude that L-658,758 is an effective inhibitor of NE, proteinase-3 and CF sputum sol elastase.

CF is characterized by chronic pulmonary infection and inflammation and a progressive loss of pulmonary function (Davis et al., 1996). It is thought that repeated cycles of infection, particularly with Pseudomonas aeruginosa, and subsequent inflammation contribute to CF lung pathology (Berger, 1991). Although the mechanisms by which inflammation induces lung injury are not fully understood, studies of the contents of CF sputum and BAL have identified a variety of neutrophil proteases and bacterial products with the potential to contribute to pulmonary disease.

In particular, high levels of elastinolytic activity have been detected in sputum and BAL from patients with CF (Fick et al., 1984; Jackson et al., 1984; Suter et al., 1984; Bruce et al., 1985; Tournier et al., 1985). Three different potential sources of elastolytic activity are present in the lungs in CF. Neutrophil secretory granules contain large quantities of the elastolytic serine proteases NE and proteinase-3 (Bieth, 1986); Pseudomonas aeruginosa produces a metalloelastase (Morihara et al., 1965; Wretlind and Pavlovskis, 1983); lung macrophages produce other matrix metalloproteinases capable of degrading elastin, including 92-kD gelatinase, matrixin and macrophage metalloelastase (Banda and Werb, 1981; Chapman and Stone, 1984; Shapiro et al., 1993).

Many studies have examined the potential of these elastases to damage the lungs and to perpetuate cycles of infection, inflammation and injury. NE, proteinase-3, human macrophage metalloelastase and Pseudomonas elastase are capable of degrading proteins of the lung extracellular matrix in vitro (Bieth, 1986; Hamadaou et al., 1987; Rao et al., 1991; Shapiro, 1994). In addition, NE, proteinase-3 and Pseudomonas elastase, when instilled directly into rodent lungs, induce injury in many forms, including hemorrhage and emphysema (Kao et al., 1988; Lucey et al., 1988; Williams et al., 1992).

Notably, when they are present in excess, both NE and Pseudomonas elastase are capable of degrading the major antiproteases of the lungs, α1-PI and SLPI, thereby reducing the lungs’ primary protection against harmful proteolysis (Baumstark et al., 1977; Morihara et al., 1979; Johnson et al., 1982; Cantin et al., 1989; Suter and Chevallier, 1991). In addition, NE can actively promote inflammation, and thus perpetuate itself in the lungs, by stimulating human epithelial cells to produce IL-8, a potent neutrophil chemottractant.
tant (Nakamura et al., 1992; Richman-Eisenstat et al., 1993). In keeping with this, levels of IL-8 in the epithelial lining fluid of CF patients are reduced by treatment with aerosolized rSLPI (McElvaney et al., 1992).

Elastases may also compromise host defenses against infection. Elastases degrade IgG and IgA, as well as the CR1 receptor on neutrophils and C3bi fragments on Pseudomonas, all of which could act to diminish Pseudomonas opsonization and killing (Fick et al., 1984; Tosi et al., 1990). Furthermore, elastases introduced into rodent lungs have been shown to increase adherence of Pseudomonas to tracheal epithelia (Woods et al., 1980; Plotkowski et al., 1989). Exposing epithelium to these elastases leads to increased secretion of mucus (Klinger et al., 1984; Christensen et al., 1987; Somerville et al., 1991), as well as to decreased ciliary beat frequency (Amitani et al., 1991).

All of these effects, if they occurred in vivo, would have serious consequences for CF patients, for whom pulmonary infection and deterioration can be life-threatening. Given the potential for injury demonstrated by elastases, an arsenal of anti-NE drugs is under development for use in disease states with potential for injury demonstrated by elastases, an arsenal of anti-NE drugs is under development for use in disease states characterized by chronic inflammation. Among these are a family of β-lactam anti-elastases, which are mechanism-based, time-dependent inhibitors of NE (Doherty et al., 1986; Davies et al., 1991). Many have been shown to be potent, specific and stable inhibitors of NE activity in vitro (Bonney et al., 1989; Knight et al., 1992a; Knight et al., 1992b).

Our goal in the studies reported here was to determine how well the cephalosporin-based anti-elastase L-658,758 blocks the activity of human neutrophil elastase, human proteinase-3, human macrophage metalloelastase, mouse macrophage metalloelastase and Pseudomonas aeruginosa elastase. In addition, we measured its ability to block elastolysis by CF sputum sol. Finally, we examined the ability of a panel of inhibitors to block the activity of elastases in CF sputum in vitro.

Materials and Methods

**CF sputum sol.** Patients for this study were adults with moderate to advanced CF who were hospitalized at Children’s Hospital (Boston, MA) for treatment of acute pulmonary exacerbation. Sputum was collected on ice after the patients’ morning chest physiotherapy. Within 4 h, it was fractionated in the cold, by centrifugation at 50,000 × g for 90 min, into a gel pellet containing cells and a sol supernatant containing extracellular enzymes (Jackson et al., 1984). Sputum sol was stored at −70°C until analysis.

**Elastases.** Proteinase-3 was prepared from lysates of leukocyte granules obtained from patients with chronic myeloid leukemia by modification of the procedure of Kao et al. (1988). Briefly, granules were extracted by sonic disruption in 15 mM HCl. After centrifugation, the supernatant fluid was chromatographed on a Dymatrix Orange A-agarose dye affinity column. Enzymatically active fractions were pooled and further purified on Trasylol-Sepharose and by ion-exchange chromatography on SP-Sepharose. The human proteinase-3 isolated by this method had little or no contamination with neutrophil elastase. The material was homogeneous as evaluated by LC electrospray-mass spectrometry, N-terminal amino acid sequence determination and SDS-polyacrylamide gel electrophoresis (manuscript in preparation).

NE was purchased from Elastin Products (Owensville, MO) and Pseudomonas elastase from Nagase Biochemicals (Tokyo, Japan). Human and mouse macrophage metalloelastases were generous gifts of Dr. Steven Shapiro.

**Elastase inhibitors.** Compounds used to inhibit elastinolytic activity were EDTA (10 mM), 1,10-phenanthroline (10 mM), α1-PI (77 μM), SLPI (0.71 μM), CMK (0.1 mM) (Enzyme Systems Products, Livermore, CA) and L-658,758 (0.1 mM) (Merck Research Laboratories, Rahway, NJ). For inhibition of individual sol samples with CMK and L-658,758 (table 2), inhibitor concentration was 0.1 mM. In other experiments, sol was incubated at 37°C for 30 minutes with varying concentrations of L-658,758, then assayed for activity towards elastin or synthetic substrate (fig. 3).

**Elastase assays.** Elastinolytic activity was determined by measuring the degradation of 3H-elastin (table 1 and fig. 1). 3H-elastin was prepared by reductive alkylation of bovine neck ligament elastin (Elastin Products, Owensville, MO) as described by Gordon et al. (Gordon et al., 1976). For assay of activity of NE, NE and pooled CF sputum sol with and without inhibitors, 2 μg of elastase was assayed in 200 μl of 0.1 M NaCl, 0.125 M MOPS, pH 7.5, containing 800 μg of 3H-elastin (1030 cpm/μg elastin). The reaction mixture was incubated for 24 h at 37°C and then centrifuged at 13,000 × g for 15 min to pellet undegraded elastin. Solubilized 3H-peptides in the supernatant were measured by liquid scintillation counting. For figures 1 and 2, 100% activity of pooled sol represents degradation of 230 μg of elastin in 24 h. Human and mouse macrophage metalloelastase were assayed in the same manner (fig. 1), except that 5 mM CaCl2 was incorporated into the assay buffer to provide optimal conditions for activity. For assays in the presence of inhibitor, elastase and inhibitor were incubated for 30 min at 37°C before the addition of 3H-elastin substrate.

For assays of individual sol samples (table 2), elastolysis was measured similarly but in a larger volume and in a buffer of higher ionic strength. A 20-μl aliquot containing 330 μg of 3H-elastin (1377 cpm/μg elastin) in 1 M NaCl, 10 mM KH2PO4, 0.015% Tween-20, pH 7.4, was combined with sol samples diluted 200-fold in 1 M NaCl, 0.125 M KH2PO4, pH 7.4, in a final volume of 0.97 ml. The assay proceeded for 24 h and was analyzed as described above. Elastinolytic activity was converted to an equivalent NE concentration by comparison with a nonlinear standard curve generated with pure active site-titrated human NE.

NE activity was also measured using a specific peptide substrate, AAPV-pNA. The change in Abs 405 nm of a reaction mixture containing sol in 1.0 M NaCl, 0.075% Tween 20, 0.125 M KH2PO4, pH 7.4, and 1 mM substrate was measured kinetically at room temperature in a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA). Standard curves, which were linear with concentration, were generated using known amounts of active site-titrated pure human NE.

For assays of pooled sol or NE with increasing concentrations of L-658,758 (fig. 3), elastase was incubated with an equal volume of inhibitor for 30 min at 37°C and then was diluted and assayed as described above.

**Statistical analyses.** Statistical analyses were performed by one-way analysis of variance (figs. 1 and 2).

**Results**

From 17 patients with CF who were undergoing i.v. antibiotic therapy to combat an acute pulmonary exacerbation, we collected 35 sputum samples. Ranging in age from 15 to 36, all these patients had moderately advanced disease. All subjects were colonized with Pseudomonas aeruginosa. Sputum samples, which averaged 9 ml, were fractionated into gel and sol phases. Then 22 samples from nine patients were pooled to prepare pooled CF sputum sol; 13 samples from eight patients were studied individually.

Three potential sources of elastase in CF sputum are the neutrophil, the macrophage and Pseudomonas. We investigated the ability of L-658,758 to block proteolysis by elastases from these sources and to block elastases in pooled CF.
Elastinolytic activity of elastases and CF sol

Table 1

<table>
<thead>
<tr>
<th>Elastase</th>
<th>Elastin Degraded (μg elastin/mg elastase/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>132.6</td>
</tr>
<tr>
<td>PR-3</td>
<td>72.8</td>
</tr>
<tr>
<td>PsE</td>
<td>147.5</td>
</tr>
<tr>
<td>CF sol**</td>
<td>177.3</td>
</tr>
<tr>
<td>5 mM</td>
<td>HME</td>
</tr>
<tr>
<td>5 mM</td>
<td>MME</td>
</tr>
<tr>
<td>5 mM</td>
<td>CF sol**</td>
</tr>
</tbody>
</table>

* Assay buffer is 0.1 M NaCl, 0.125 M MOPS, pH 7.4, with or without addition of 5 mM CaCl₂.
** Elastase concentration in CF sol is taken to be the NE concentration measured by hydrolysis of AAPV-pNA.

Discussion

This study demonstrates that the cephalosporin-based compound L-658,758 is an effective inhibitor of the purified elastases NE and proteinase-3, as well as of the elastinolytic activity of CF sputum. Human and mouse macrophage metalloelastase and Pseudomonas elastase were not inhibited by L-658,758. Sputum from CF patients was found to contain high levels of elastinolytic activity. This activity could in...
large part be attributed to neutrophil elastase, because it was blocked by inhibitors of NE (α1-PI, SLPI, CMK, and L-658,758) but not by inhibitors of metalloelastases.

A number of studies have addressed the question of which types of elastases are present in CF airway secretions. Although some have suggested the presence of modest amounts of Pseudomonas elastase (Fick et al., 1984; Bruce et al., 1985), most have concluded that NE is the predominant elastase in CF airway secretions (Jackson et al., 1984; Suter et al., 1984; Tournier et al., 1985). The results of the present study are in keeping with these. However, the observation that a low level of sol elastase activity persisted in the presence of SLPI raises the possibility that a trace of active proteinase-3 may be present in CF sol.

**TABLE 2**

NE activity in individual samples of CF sputum sol

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Elastin Degraded** (µg/ml sol/min)</th>
<th>NE** (µM)</th>
<th>Elastin Degraded (% of total) with CMK</th>
<th>Elastin Degraded (% of total) with L-658,758</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>10.1</td>
<td>4.72</td>
<td>3.1</td>
<td>0.0</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>3.2</td>
<td>0.47</td>
<td>2.3</td>
<td>0.0</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>12.6</td>
<td>6.70</td>
<td>3.6</td>
<td>0.0</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>13.0</td>
<td>7.02</td>
<td>3.0</td>
<td>1.1</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>11.1</td>
<td>5.50</td>
<td>2.1</td>
<td>0.0</td>
</tr>
<tr>
<td>E</td>
<td>7</td>
<td>9.6</td>
<td>4.30</td>
<td>2.3</td>
<td>0.0</td>
</tr>
<tr>
<td>E</td>
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<td>1.3</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>14.6</td>
<td>8.24</td>
<td>2.3</td>
<td>0.0</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>8.5</td>
<td>3.49</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>F</td>
<td>11</td>
<td>6.7</td>
<td>2.05</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>G</td>
<td>12</td>
<td>26.3</td>
<td>18.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>H</td>
<td>13</td>
<td>4.2</td>
<td>1.20</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Elastinolytic activity was measured with [3H]-elastin substrate in 1.0 M NaCl, 0.075% Tween 20, 0.125 M KH2PO4, pH 7.4.
** Elastinolytic activity was converted to an equivalent NE concentration by comparison with a standard curve generated with human NE and reflects the assumption that all sol elastinolytic activity derived from NE.

As predicted from their mechanisms of action, none of the antielastases with activity toward NE and proteinase-3 inhibited the metalloelastases, human and mouse metalloelastase and Pseudomonas elastase. Therefore, treatment with currently available NE inhibitors will not block proteolysis by human macrophage metalloelastase. Human macrophage metalloelastase is a potent protease that, although it does not appear in measurable quantities in CF sputum, may yet contribute to lung destruction in diseases such as CF and emphysema by acting locally at sites of macrophage binding.

The high levels of active NE in CF sputum sol demonstrate that important antiprotease defenses have been overwhelmed in the CF patient’s airways and highlight the potential for extensive lung damage through proteolysis. Elevated elastinolytic activity in CF airway secretions can begin early in a patient’s life (Birrer et al., 1994; Khan et al., 1995) and parallels the severity of lung disease (Suter et al., 1984; O’Connor et al., 1993). In a direct demonstration of the toxicity of elastase in CF airway secretions, CF sputum sol was shown to induce lung injury and inflammation when introduced into the lungs of healthy rats by intratracheal instillation (Rees and Brain, 1995). The hemorrhagic component of the observed injury was prevented by p.o. pretreatment of the rats with antielastase. These observations, combined with our knowledge of the variety of soluble and insoluble substrates that NE and proteinase-3 have been shown to degrade in vitro and of the injury they have been demonstrated to induce in animal models in vivo, all argue that reduction of elastase activity in the CF patient’s lungs may prove beneficial (Bieth, 1986; Kao et al., 1988; Lucey et al., 1988; Rao et al., 1991).

Therefore, we further examined the ability of L-658,758 to reduce elastase activity in individual samples of CF sol. L-658,758 inhibited CF sol elastinolysis virtually completely, with less than 1% of activity remaining in most samples. The concentration of L-658,758 required to achieve 50% inhibition varied with the concentration of NE present in the reaction mixture. This is consistent with its reported mech-
anism as a time-dependent, irreversible inhibitor that ti-
trates the active site of the enzyme. Approximately 5-fold
molar equivalents of inhibitor were sufficient to achieve 50%
inhibition, a result that demonstrates relatively little reac-
tion with water or other components of the reaction mixture.
The low concentrations of inhibitor required to achieve
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spumt sol suggest that L-658,758 or a related compound
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References


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